AD NUMBER

AD469219

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BDRL, D/A ltr, 28 Sep 1971

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TECHNICAL MANUSCRIPT 236

DIRECT FLUORESCENT TAGGING OF MICROORGANISMS: POSSIBLE LIFE DETECTION TECHNIQUE

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Project 1C622401A071

August 1965

ACKNOWLEDGMENTS

The authors express their appreciation to Mr. Frank W. Wagner for furnishing atmospheric background samples, and to Mr. Franklin T. Wetzel for cover slip preparations of hamster kidney cells. Thanks are also extended to Dr. John M. Ladino for micro-Kjeldahl determinations and to Dr. Sidney Yaverbaum for helpful suggestions during the course of this study. The technical assistance of Lt. Joseph N. Freund and Sp-4 Robert J. Bammer, Jr. is gratefully acknowledged.

ABSTRACT

Microorganisms and selected proteinaceous substances were directly tagged with fluorescein isothiocyanate. This approach suggested a possible application for detection of extraterrestrial life. A stable and apparently specific linkage was formed with protein, although non-protein substances were readily destained. Substances such as soil and atmospheric debris did not exhibit any significant affinity for the dye.

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DIRECT FLUORESCENT TAGGING OF MICROORGANISMS: A POSSIBLE LIFE DETECTION TECHNIQUE

During experimental investigations on methods for tagging bacterial cells with fluorescent labels, it was found that microbial protein could be rapidly conjugated with fluorescein isothiocyanate (FITC). The simplicity and apparent specificity of the technique suggested a possible approach for detecting extraterrestrial life.[1] The principal sources of biological material are often presumed to be soil and atmospheric debris. Some of the methods proposed for detecting extraterrestrial life are based on the assumption that microorganisms may be a predominant form of life and consequently systems have been developed for detecting bacteria or their metabolic products. These systems depend on integrated metabolic reactions and growth of microorganisms. The use of simplified approaches may be more realistic when applied to unknown extraterrestrial environments. However, all approaches are, of necessity, based on certain assumptions, and ultimately, a combination of different methods may be required for a definitive answer.

This preliminary report describes a simple staining procedure that is based on direct conjugation of FITC with protein particulates. Basically, the method involves the conjugation of small concentrations of protein with FITC and approximates the conditions for labeling protein in the fluorescent antibody technique.[2-4] A stable linkage with protein appears to be formed; nonproteinaceous substances are readily destained. In comparison with fluorescent antibody, the direct conjugation of nonantibody protein has received limited application in experimental studies. However, a variety of proteins have been conjugated and these include soluble bacterial antigens,[5] virus particulates,* bovine albumin,[6] hormones,[7] enzymes,[8] and amino acids.[9] The present study was concerned with establishing an experimental basis for utilizing protein conjugation with FITC as a rapid and nonspecific method of biological detection.

Three classes of representative substances were utilized to evaluate staining reactions with FITC. These consisted of (i) bacteria and selected protein-type substances; (ii) nonprotein biochemical substances; and (iii) predominantly inorganic materials that included shelf compounds, atmospheric debris, and soil samples.

All test substances were examined as smear preparations on standard microscopic slides. Smears of individual and combined test materials were initially prepared in distilled water droplets. Bacterial suspensions were adjusted to contain approximately 10^8 cells per ml. Concentrated atmospheric debris was collected by impaction on glass slides and examined with and without added organisms. Cover slip preparations of hamster kidney cells were stained directly and mounted.

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* Dr. James E. Smith, Syracuse University, personal communication.

A standard staining solution of FITC* was prepared with the following reagents: 1.3 ml of 0.5 M carbonate-bicarbonate buffer (pH 9.6); 6.0 ml of 0.01 M phosphate-buffered saline (pH 7.2);* 5.7 ml of 0.85% physiological saline; and 5.3 mg of crystalline FITC** added as a dry powder. After mixing on a Vortex mixer for 30 minutes at ambient room temperature, the solution was centrifuged at 3000 rpm for 20 minutes to remove any insoluble particulates. Preparations were either used immediately or stored in the dark at 4 C for no longer than 6 hours.

Duplicate air-dried and gently heat-fixed smears were covered with 0.1 ml of FITC solution and stained in a moist chamber. Bacteria were treated for one minute at 37 C; other substances were stained for 20 minutes at room temperature. Bacterial smears were warmed to 37 C prior to staining at this temperature. Following staining, slides were rinsed and washed for 10 minutes in 0.5 M carbonate-bicarbonate buffer at pH 9.6 and mounted in glycerol adjusted to pH 9.6 with 2% carbonate-bicarbonate buffer. Unstained controls were treated in the same manner. Slides were examined by fluorescence microscopy as described in a previous publication.[10]

Experimental evidence for firm binding of FITC to bacterial protein was obtained by passage of conjugated cells through a Sephadex column and by acetone treatment of stained cells. Two comparable cell suspensions were prepared in the following manner. Six ml of a phosphate-buffered saline suspension of Serratia marcescens (10^9 cells per ml) were combined with the standard conjugation reagents and mixed for 30 minutes at room temperature. The staining intensity of each preparation was examined after this treatment.

Conjugated cells from one preparation were passed through a G-25 Sephadex column (coarse, nonbeaded, 28 cm x 2.5 cm) equilibrated and eluted with 0.5 M carbonate-bicarbonate buffer (pH 9.6). Five fractions were collected separately in conical centrifuge tubes. After centrifugation for 20 minutes at 3000 rpm, smears were prepared from the sediment of each tube, allowed to dry, and then mounted in buffered glycerol. All fractions were examined for fluorescence intensity of stained cells.

After conjugation, the second suspension was centrifuged at 3000 rpm for 20 minutes. The pellet was washed with cold phosphate-buffered saline (pH 7.2) and finally suspended in one ml of buffered saline. Eight ml of cold acetone were added and the contents mixed for several minutes. After mixing, the suspension was centrifuged and washed with buffered saline. Acetone extraction was repeated for a second time and the bacterial pellet

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* Reagents and volumes used for the standard FITC solution approximate the conditions for conjugating 130 mg of antibody protein in the fluorescent antibody method. The antibody protein to be conjugated is usually dissolved in phosphate-buffered saline.

** Obtained from Baltimore Biological Laboratories.

was finally suspended in one ml of 0.5 M carbonate-bicarbonate buffer (pH 9.6). A drop from the suspension was placed on a slide, air-dried, and mounted in buffered glycerol.

The effect of pH on the FITC staining reaction was evaluated at two different pH levels. Staining at pH 7.2 was performed with an FITC solution containing 7.3 ml of buffered saline (pH 7.2), 5.7 ml of physiological saline, and 5.3 mg of FITC Staining at pH 9.6 was conducted with the standard FITC solution. Smears of S. mercescens were prepared on slides and prewarmed to 37 C. Staining solutions were applied for one minute at 37 C. After staining, slides were rinsed and washed in acetone for 15 minutes. Acetone-treated slides were again washed in distilled water for 5 minutes and given a final wash in 0.5 M carbonate-bicarbonate buffer (pH 9.6). Smears were mounted in buffered glycerol.

The specificity of the FITC reaction was further evaluated by staining preparations of Bacillus anthracis, soil, and atmospheric debris with FITC solution and normal rabbit globulin conjugated with FITC. Additional specificity studies included (i) use of a staining solution containing sodium fluorescein instead of FITC, and (ii) complete blocking of FITC staining by pretreatment of bacterial cells with 2,4,dinitrofluorobenzene for 24 hours.

The possible existence of protein impurities in sodium glycerophosphate was determined by a modified Biuret reaction[11] and absorbance at 280 mμ. A micro-Kjeldahl procedure was used for determining the protein content of glycogen.

Experimental data have shown that selected proteins were stained, nonprotein biochemical substances as well as inorganic compounds were destained (Tables 1 and 2). However, such substances as glycogen, impure RNA, and sodium glycerophosphate exhibited some staining to a greater or lesser degree (Table 2). The latter results may be explained on the basis of protein contamination, which in turn would depend on origin of starting materials (e.g., animal glycogen or fat) and degree of purification. For example, sodium glycerophosphate and glycogen were found to contain a significant amount of protein-type impurity (Table 2).

The critical differentiation between atmospheric background and specific staining of bacterial protein was evaluated with standard FITC solution. The results have shown that background was readily destained, but that particles of possible biological origin were brightly stained. This effect was strikingly more apparent when bacterial cells were added to atmospheric debris and soil (Table 3). Figure 1 illustrates staining of B. anthracis in atmospheric debris.

TABLE 1. STAINING OF SELECTED PROTEINS AND BACTERIA WITH STANDARD FITC SOLUTION[a]

Test Substance	Staining Intensity[b]	Unstained Control
Protein-Polypeptide-Amino Acid		
Egg Albumin	4+	-
DL-Cysteine·HCl	2+	-
Glycyl-L-Tyrosine	3+	-
Hamster Kidney Cells	4+	-
Mouse Liver Powder	4+	-
Wheat Germ	4+	-
Bacteria		
Bacillus anthracis CD-3S	4+	-
Bacillus anthracis (spores)	4+	-
Bacillus subtilis var. *niger*	4+	-
Brucella abortus 6NIH-1	3+	-
Listeria monocytogenes ATCC7644	4+	-
Salmonella typhosa ATCC9992-V	4+	-
Serratia marcescens 8UK	4+	-

a. Protein-polypeptide-amino acid group stained for 20 minutes at room temperature; bacteria stained for one minute at 37 C.

b. Estimated brightness based on minimal to maximal intensity (1+ to 4+); - = no staining.

TABLE 2. STAINING OF NONPROTEIN BIOCHEMICAL SUBSTANCES AND INORGANIC COMPOUNDS WITH STANDARD FITC SOLUTION[a/]

Test Substance	Staining Intensity[b/]	Unstained Control
Nonprotein Biochemical Substances		
Glycogen	±[c/]	-
DNA (impure)	-	-
RNA (impure)	2+	-
DNA (pure)	-	-
Sodium Glycerophosphate	2+[c/]	-
Inorganic Substances		
Zinc Powder	-	-
Stannous Chloride	-	-
Potassium Permanganate	-	-
Mercuric Chloride	-	-
Molybdenum Trioxide	-	-

a. Test substances stained for 20 minutes at room temperature.

b. Estimated brightness based on minimal to maximal intensity (1+ to 4+); ± = several fluorescent particles observed in each microscopic field; - = no staining.

c. Glycogen and sodium glycerophosphate contained 0.6 and 0.04% by weight of protein impurity respectively; the concentration of protein impurity in DNA and RNA was not determined.

TABLE 3. STAINING OF ATMOSPHERIC AND SOIL BACKGROUND AS WELL AS ADDED TEST ORGANISMS WITH STANDARD FITC SOLUTION[a]

Test Substance	Staining Intensity[b]	Unstained Control
Soil[c]	±	-
B. anthracis in Soil	4+	-
Atmospheric Debris[c]	±	-
B. anthracis in Air Debris	4+	-
B. subtilis var. *niger* (spores) in Air Debris	3+	-
B. abortus in Air Debris	3+	-

a. Test substances stained for 20 minutes at room temperature.
b. Estimated brightness based on minimal to maximal intensity (1+ to 4+); ± = an average of one and six brightly stained particles recorded in 30 microscopic fields for atmospheric and soil background respectively (some stained particles in soil resemble rod-like bacteria); - = no staining.
c. A total of 6 soil samples and 12 air samples were examined.

Figure 1. Differentiation of FITC-Stained Cells of B. anthracis from Atmospheric Background. Organisms stained bright apple-green and background blue on high-speed Ektachrome film (120 seconds).

Staining reactions illustrating the specificity of FITC solution as compared with FITC-conjugated normal rabbit globulin are presented in Table 4. Experimental results have shown that FITC conjugated with protein was not available for coupling with unlabeled protein (bacterial protein). Specificity was dependent on the presence of proper protein combining groups[12] and available FITC. When sodium fluorescein was substituted for FITC no specific fluorescence was observed. This could be expected, since an isothiocyanate linkage was not present for coupling with protein. Pretreatment of bacterial cells with 2,4,dinitrofluorobenzene completely inhibited staining with FITC and indicated effective blocking of combining sites on the bacterial protein.

TABLE 4 STAINING SPECIFICITY OF FITC SOLUTION COMPARED WITH FLUORESCEIN-LABELED NORMAL RABBIT GLOBULIN[a/]

Test Substance	Staining Intensity[b/]	
	FITC	Labeled Normal Rabbit Globulin
Atmospheric Debris	±	-
B. anthracis	4+	-
Soil	±	-
B. anthracis in Soil	4+	-

a. Test substances stained for 20 minutes at room temperature.
b. Estimated brightness based on minimal to maximal intensity (1+ to 4+); - = no staining; ± = occasional fluorescent particles of possible biological origin.

The effect of acetone extraction or passage through a Sephadex column on the linkage of FITC to protein is shown in Table 5. The intensity of fluorescence following such treatment did not appear to be affected, and indicated firm binding of FITC to bacterial protein. The staining reactions of S. marcescens smears treated with solutions buffered at pH 7.2 and pH 9.6 are also presented in Table 5. They show that the fluorescence intensity of preparations stained at pH 7.2 is markedly reduced and implicates rapid conjugation at a higher pH (9.6) as the factor responsible for brighter staining.[3,18]

TABLE 5. DETERMINATION OF BINDING OF FITC TO BACTERIAL PROTEIN (S. MARCESCENS)

Experiment	Method of Staining[a]	Method for Determining Firmness of FITC Binding	Staining Intensity of Treated Cells[b]
1	Cells stained in solution at pH 9.6	Passage through Sephadex column	4+
2	Cells stained in solution at pH 9.6	Acetone extraction	4+
3	Cells stained on a slide at pH 9.6	Acetone extraction	4+
4	Cells stained on a slide at pH 7.2	Acetone extraction	2+

a. Cells were stained in standard conjugation solution for 30 minutes at room temperature; a one-minute staining period at 37 C was used for slide preparations.

b. Estimated brightness was based on minimal to maximal intensity (1+ to 4+); staining intensity remains unaffected following Sephadex and acetone treatment.

The staining technique described in this report was simple and did not require any unusual manipulations. Specific staining appeared to depend on the availability of proper combining groups as well as the usual conditions for conjugating protein. A wider latitude in conjugation methodology is possible with bacterial and other nonantibody proteins, because conditions that may affect antibody combining activity are of little concern in the present study.

It is impossible, of course, to predict the reactions of FITC in extraterrestrial environments. Two major sources of error might be encountered: (i) reaction of FITC with nonprotein substances of unusual chemical configuration; and (ii) emission of green autofluorescence by nonprotein materials.

For satellite probes, the FITC method could be used in the following manner. Soil or dust particles are drawn into a previously filtered FITC solution and reacted for several minutes. The reaction mixture is passed through a filter-type membrane and stained particles are deposited on the surface. The membrane surface is given a buffer wash and particles are then scanned with a sensitive phototube device.

Solutions of FITC buffered at pH 9.6 tend to become unstable after several hours. Therefore, for extended missions, FITC powder could be mixed with liquid reagent at the time of sampling. Instrumentation of the FITC reaction should not be any more complex than that proposed for present systems.

The FITC method is based on the assumption that proteinaceous substances (particularly microorganisms) may exist in extraterrestrial environments and possess structural characteristics that are reminiscent of Earth protein.

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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1 ORIGINATING ACTIVITY (Corporate author)

U.S. Army Biological Laboratories
Fort Detrick, Frederick, Maryland, 21701

2a REPORT SECURITY CLASSIFICATION

Unclassified

2b GROUP

3 REPORT TITLE

DIRECT FLUORESCENT TAGGING OF MICROORGANISMS: A POSSIBLE LIFE DETECTION TECHNIQUE

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5 AUTHOR(S) (Last name, first name, initial)

Pital, Abe NMI
Janowitz, Sheldon L.
Hudak, Charles E.
Lewis, Evelyn E.

6 REPORT DATE	7a TOTAL NO OF PAGES	7b NO. OF REFS
August 1965	18	13

8a CONTRACT OR GRANT NO	9a ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO 1C622401A071	Technical Manuscript 236
c.	9b OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10 AVAILABILITY/LIMITATION NOTICES

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11 SUPPLEMENTARY NOTES	12 SPONSORING MILITARY ACTIVITY
	U.S. Army Biological Laboratories Fort Detrick, Frederick, Maryland, 21701

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Microorganisms and selected proteinaceous substances were directly tagged with fluorescein isothiocyanate. This approach suggested a possible application for detection of extraterrestrial life. A stable and apparently specific linkage was formed with protein, although nonprotein substances were readily destained. Substances such as soil and atmospheric debris did not exhibit any significant affinity for the dye.

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